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Sequence Analysis of Bacterial DNA in the Colon of an Andean Mummy

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ABSTRACT We have isolated DNA from 14 tissue samples from the internal organs of an Andean human mummy (10th–11th century A.D.) and have checked the persistence of the original human and bacterial templates using the following main approaches: 1) amino acid racemization test; 2) quantification of mitochondrial DNA copy number; 3) survey of bacterial DNA in the different organs; 4) sequence analysis of bacterial amplicons of different lengths. The results demonstrate that both the original human DNA and the DNA of the bacteria of the mummy gut are preserved. In particular, sequence analysis of two (respectively 100 and 196 bp in length) libraries of bacterial 16s ribosomal RNA gene amplicons from the mummy colon shows that while the shortest amplicons give only modest and biased indications about the bacterial taxa, the longer amplicons allow the identification several species of the genus *Clostridium* which are typical of the human colon. This work represents a first example of a methodological approach which is applicable, in principle, to many other natural and artificial mummies and might open the way to the study of the structure of the human microbial ecosystem from prehistory to present. Am J Phys Anthropol 107:285–295, 1998.

The molecular studies on bacteria and other microorganisms in ancient human remains performed to date have mainly addressed typing and characterization of pathogen DNA. In particular, *Mycobacterium tuberculosis* DNA has been detected in Renaissance (Faerman et al., 1997), Medieval (Salo et al., 1994; Dixon et al., 1995), and Predynastic (Crubèzy et al., 1997) skeletons and mummies. Archaeomolecular investigations have also considered *Mycobacteriun leprae* (Rafi et al., 1994), *Yersina pestis* (Hummel et al., 1994), *Treponema pallidum* (Rogan and Lentz, 1995; Marota et al., 1995), and a sole example of a non-pathogenic microorganism, *Escherichia coli* (Fricker et al., 1997).

On the other hand, there have been only few attempts to perform a broad-range description of the microbial DNA in a human mummy. Rollo et al. (1994, 1995) analyzed the grass clothing of the so-called Tyrolean iceman (5300–5100 years B.P.) and detected the DNA of a number of microorganisms including microalgae, filamentous fungi, and yeasts. The same authors used sequence analysis of microbial DNA to challenge the hypothesis (Haselwandter and Ebner, 1994)

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that the iceman's boots could host anabiotic (i.e., capable of returning to life) prehistoric fungi (Ubaldi et al., 1996).

All external body surfaces have a normal resident bacterial flora, and this includes the digestive tract. The indigenous microbiota play an important role in health and diseases of humans and animals. They contribute to the development of the immune system and provide resistance to colonization by allochtonous or pathogenic microorganisms. They also constitute a reservoir of potentially pathogenic bacteria that may infect host tissues (Savage, 1977; Marcotte and Lavoie, 1998). In particular, studies performed during the past decades have given an appreciation of the complexity of the colonic microbial flora of humans. Such information has come mainly from analyses of faecal samples. There is also some limited information on the colonic flora in various populations around the world. The human gut does not have a true mucosal flora analogous to that of the chicken gut (with its palisades of lactobacilli) or the rat (with its spiral flora). Nevertheless, it has a flora associated with the mucosa which is distinct from that of the gut lumen and which is reproducible and stable (Hill, 1995).

We have investigated the possibility of collecting historical data on the composition of the bacterial flora of the colon by examining a pre-Columbian mummy from Cuzco, Peru. The mummy, presently at Museo Nazionale di Antropologia ed Etnologia, Florence, Italy, is of a young woman aged 20 ± 3 years. A few years ago the Andean mummy was examined by Fornaciari et al. (1992) and the internal organs, stomach, lungs, intestine, liver, heart, and other tissues were found to be in a good state of preservation.

The study of the ancient microbial DNA preserved in the intestines of this mummy has allowed us to set up a general research protocol that is directly applicable to other mummies and might offer, among other information, interesting insights into the health and dietary habits of human populations through time.

MATERIALS AND METHODS

The Andean mummy

This mummy (catalogue number 3076, Museo Nazionale di Antropologia ed Etnologia, Florence) originally belonged to the "Mazzei" collection, which was later dismantled and preserved in part at the Museo Nazionale di Antropologia ed Etnologia and in part at the Museo Etnografico e Preistorico "L. Pigorini" (Rome). On the basis of the funerary goods, the mummy was dated to the 14th–15th century A.D. (Laurencich-Minelli, 1993). Radiocarbon analyses recently performed at the "Pigorini" museum (R. Machiarelli, personal communication), however, suggest that all the mummies from the "Mazzei" collection could be considerably older than previously thought: they may date to the 10th–11th century A.D.

The tissue samples utilized for DNA extraction were collected during the necropsy performed in 1992 by G. Fornaciari and associates (Fornaciari et al., 1992).

Laboratory analysis

Aspartic acid racemization. Two small fragments of myocardium (10 mg) and colon (12 mg) were washed in HCl (0.01 N) and hydrolyzed in twice-distilled 6 N HCl for 24 h at 100°C. The hydrolysates were essiccated under vacuum and the residues reconstituted in twice-distilled water. The amino acids were derivatized with O-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) and analyzed by a high-pressure liquid chromatography (HPLC) system (HP 1100, Hewlett Packard, Palo Alto, CA) using fluorescent detection (Zaho and Bada, 1995).

DNA extraction. The details of the phenol-based DNA extraction procedure have been reported previously (Rollo et al., 1994). The samples were left overnight in an extraction medium composed of sodium dodecyl sulfate (SDS), Na_2EDTA, Tris-HCl (pH 8.0), and phenol. Each tissue sample (approx. 100 mg) was then milled in a mortar with a pestle in the presence of the extraction medium. The homogenate was then extracted stepwise using phenol, phenol/chloroform, chloroform, and ether, and the DNA finally precipitated using ethanol at −20°C. The DNA was further purified by a MerMaid kit (BIO 101, Vista, CA) to eliminate PCR inhibitors. To check the size of the DNA fragments, the final preparations were analyzed by agarose gel electrophoresis (2.5% agarose).

PCR amplification of DNA. Enzymatic amplifications by PCR were performed in 50 µl of a reaction mixture containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 µM (each) dNTPs, 300 ng of each oligonucleotide primer, 2.5 units *Taq* polymerase, and 1 µl DNA. The following universal primers for the 16s ribosomal RNA gene (16s rDNA) were employed: 29f: 5′ -TGGCTCAGATTGAACGCTG-3′; 98r: 5′-CCAGACATTACTCACCCGTCC-3′; 338f: 5′-AACTGAGACACGGTCCAGAC-3′; 531r: 5′-ACGCTTGCACCCTCCGTATT-3′.

An initial step at 94°C for 5 min was carried out to denature genomic DNA. The denaturation step was set at 94°C for 60 s. The annealing step was carried out for 30 s at 60°C for the primers 29f-98r and at 56°C for the primers 338f-531r. The elongation step was at 72°C for 60 s. Forty amplification cycles for each amplification reaction were performed. To eliminate small amounts of *E. coli* DNA possibly present in the commercial enzyme preparations, and, in general, any types of contaminant DNA, the *Taq* polymerase and the reaction components were pretreated with 3 units of DNase I (from bovine pancreas) for 30 min at room temperature. After incubation, the DNase I was inactivated by boiling for 10 min at 94°C.

Precautions to avoid contamination. Tissue samples to be used for DNA extraction were prepared from whole internal organs removed during the necropsy of the mummy and which had been stored aseptically. In general, we discarded the outermost portions of the specimens in order to eliminate contamination, possibly due to sample manipulation.

In the course of DNA extraction and PCR amplification steps, we routinely employed the standard precautions for ancient DNA work (wearing of sterile gloves, pretreatment of mortars, pestles, and homogenizers with HCl, use of UV-irradiated safety cabinets, dedicated gel trays, tanks, etc.). We also implemented the different steps in physically separated laboratories and used a DNA-free chamber (Herrmann and Hummel, 1994).

Traditional contamination controls are represented by mock extractions and blank amplification reactions. We performed both types of control to check for the presence of modern human mitochondrial DNA and bacterial DNA. In our laboratories, however, monitoring of contaminant DNA does not stop at this point as we clone amplified DNA and sequence large number of bacterial amplicons. In this way we are, in principle, able to detect low copy number contaminant DNA species which might escape detection by other means. The effectiveness of the precautions adopted is shown by the fact that despite all the cloning work in our laboratories being performed using *E. coli* as the recipient organism, not a single sequence attributable to this bacterium was found in over a hundred 16s rDNA amplicons screened in the course of the present investigation.

As a final cautionary note, it may be worth adding that our sequence results were determined through the screening of 16s rDNA amplicon libraries which, in turn, were obtained following PCR amplification of a selected ancient DNA fraction (transverse colon fraction). This fraction is characterized by the presence of low molecular weight bacterial DNA at a very high multiplicity (approximately 3,000 copies per reaction mixture), as assessed by competitive PCR (see below). The high multiplicity of the ancient template prevents laboratory contaminants (if any) from entering the reaction.

DNA cloning and sequencing. Amplification products were cloned using a pMOS-Blue T-vector kit (Amersham) according to the manufacturer's instructions. Plasmid DNA was extracted using an alkaline lysis based method followed by silica gel purification (Plasmix, Talent). Sequencing was performed by the use of a Dye Terminator Cycle Sequencing Kit/AmpliTaq FS (PE Applied Biosystems, Foster City, CA). Cycle sequencing products were subsequently purified by Centri-sep spin columns (Princeton Separations, Adelphia, NJ) and analyzed using ABI-PRISM 310 and 377 DNA Sequencers (both from Perkin-Elmer).

Analysis of DNA sequences. Chimeric sequences were detected using the Check Chimera program available from the Ribosomal Database Project (Maidak et al., 1997).

Comparisons with reference sequences in GenBank and in EMBL data library were performed using, respectively, the BLAST (Altschul et al., 1990) and the FASTA (Pearson and Lipman, 1988) search programs. To establish phylogenetic correlations among ancient and modern bacterial species the 16s rDNA nucleotide sequences were aligned using the Clustal V function (Higgins, 1992). The phylogenetic tree was constructed using the Treecon (Van de Peer and De Wachter, 1993) program. In particular, the distances were corrected according to Tajima and Nei (1984) and the dendrogram was calculated using the neighbor-joining method (Saitou and Nei, 1987).

Quantitative PCR tests. To quantify the number of mtDNA molecules in the mummy tissue extracts, we employed a competitive PCR (cPCR) system based on the amplification of a short (103 bp) mtDNA region in the presence of a competitor molecule 81 bp in length (Handt et al., 1996). The competitor was prepared using the protocol of Förster (1994) as reported by Handt et al. (1996) on the basis of the nucleotide sequence (positions 16191 to 16429) of the human hypervariable region I of the mitochondrial control region (Anderson et al., 1981) but with a deletion (from position 16210 to 16231) of 22 bases. The construction (final length 216 bp) was subsequently cloned into a pMOSBlue T-vector and sequenced. The concentration of the plasmidial DNA was assessed by fluorimetry, 260/280 nm spectroscopy, and densitometry following gel electrophoresis. The competitive PCR assay was performed by mixing constant amounts of mummy DNA with known numbers (10; 100; 1,000; 10,000) of competitor and by submitting the different mixture to enzymatic amplification using the L16209/H16271 primer pair (Handt et al., 1996). Under these conditions, the mummy mtDNA produced a 103 bp band while the competitors produced an 81 bp band. Each assay was repeated at least twice.

Quantitation of low molecular weight (196 bp) bacterial 16s rDNA by competitive PCR was performed according to the above principles, but using as a competitor a fragment (232 bp in length) of *Variovorax paradoxus* 16s rDNA which had previously been PCR amplified using the 338f/531r primer-pair and cloned into a pMOSBlue T-vector.

RESULTS

DNA extraction

Samples of oesophagus, stomach, pylorus, small intestine, ascending colon, transverse colon, descending colon, liver, left and right lung, diaphragm, pericardium, myocardium, and aorta of the mummy (14 in total) collected during necropsy (Fornaciari et al., 1992) were utilized for DNA extraction. The outcome of the electrophoretic fractionation of DNA is shown in Figure 1. It can be observed that all the extracts contain DNA fragments ranging in length from a few dozen base pairs to approximately 200 bp. Aorta, pericardium, myocardium, oesophagus, and transverse colon give the maximum DNA yield as shown by the more intense brightness of the smears. On the other hand, the complete absence of high molecular weight bands in the gel picture indicates that the mummy tissues do not contain intact microbial DNA and, therefore, recent microorganisms in an appreciable amount.

Quantitative PCR tests

To test the degree of conservation of the original templates we initially employed two standard tests: evaluation of the extent of racemization of aspartic acid and quantification of mitochondrial DNA (mtDNA) copy number.

Some amino acids such as aspartic acid have a racemization rate (i.e., transition from L- to D-enantiomers) similar to the DNA depurination rate. Amino acid racemization can thus be used as an indirect method to assess the extent of DNA preservation in an ancient specimen (Poinar et al., 1996). The estimation of aspartic acid racemization level performed on myocardic and colon tissue samples provides a value for the D/L ratio fully comparable to the ratio shown by fresh amino acid preparations (0.035). This result is, in principle, compatible with the preservation of the original mummy DNA.

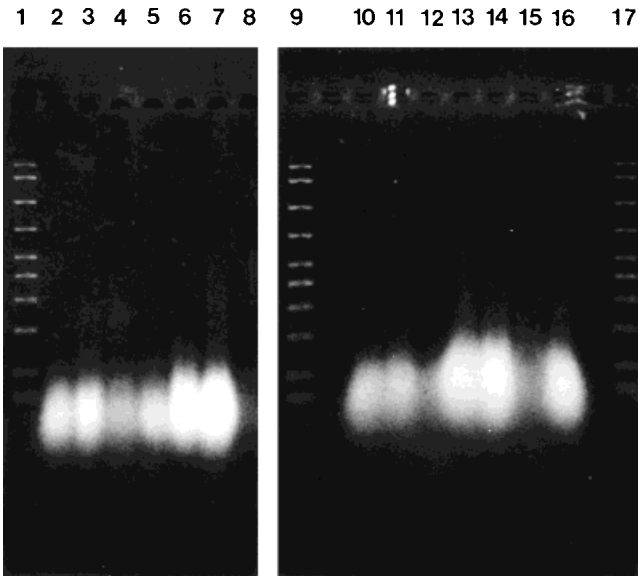

Fig. 1. Electrophoretic separation (2.5% agarose) of the DNA isolated from 14 tissue samples from the Cuzco mummy. Lanes 1, 9, and 17, molecular weight standard (50–2,000 bp ladder); lane 2, right lung; lane 3, diaphragm; lane 4, small intestine; lane 5, liver; lane 6, aorta; lane 7, pericardium; lane 8, pylorus; lane 10, descending colon; lane 11, left lung; lane 12, stomach; lane 13, transverse colon; lane 14, oesophagus; lane 15, ascending colon; lane 16, myocardium.

Recent works (Handt et al., 1993, 1994, 1996) suggest that quantitative PCR can be employed to estimate mtDNA copy number in a mummified human tissue and that this estimate can be used as an additional validation criterion for ancient DNA. We set up a competitive PCR system according to Förster (1994) to measure mtDNA copy number in several specimens from our mummy. This system is based on the amplification of a short (103 bp) tract of the hypervariable region I of the mtDNA control region (Anderson et al., 1981) in the presence of a competitor of 81 bp in length. The results showed that all the different tissue samples contained mtDNA in relatively high copy number: oesophagus approximately 100 copies; colon and myocardium approximately 200 copies. The highest figure (600 copies per reaction mixture) was shown by the aorta (Fig. 2).

A further test using a primer pair (29f/98r) designed to bind to a very short portion (approximately 100 bp) of bacterial 16s rDNA (Woese, 1987), according to the principle of the so-called (consensus sequence PCR((Relman, 1993), is performed to check the distribution of a broad range of low molecular weight bacterial DNAs within the mummy

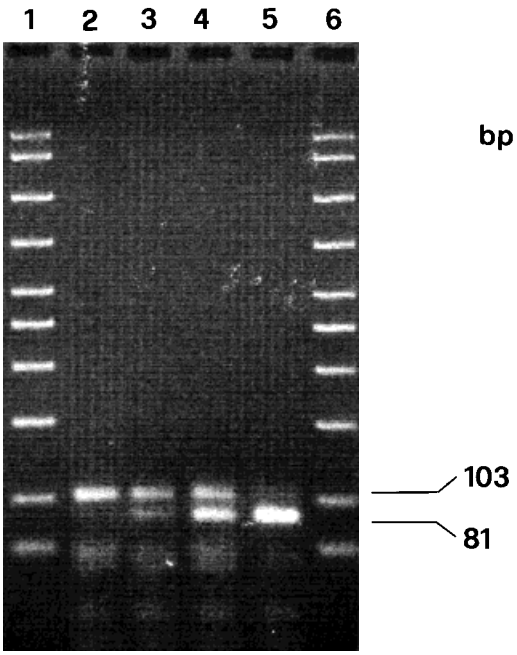

Fig. 2. Competitive PCR test to estimate mtDNA copy number (aortic tissue). Lanes 1 and 6, molecular weight standard (50–2,000 bp ladder, BioRad); lane 2, no competitor; lane 3, 100 copies of competitor; lane 4, 1,000 copies of competitor; lane 5, 10,000 copies of competitor. Electrophoresis on 3% agarose. Relative band intensity was determined by densitometry.

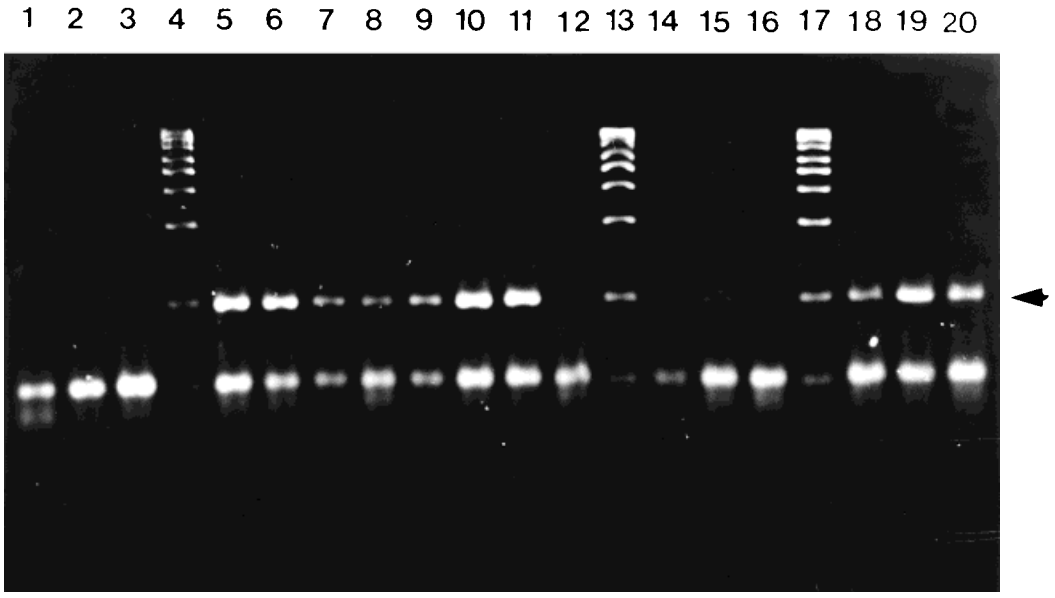

Fig. 3. PCR amplification of 14 mummy DNA samples using the primer pair 29f/98r. Lane 1, extraction blank; lanes 2 and 3, amplification controls (no DNA); lanes 4, 13, and 17, molecular weight standard (50–2,000 bp ladder, BioRad); lane 5, oesophagus; lane 6, stomach; lane 7, pylorus; lane 8, small intestine; lane 9, ascending colon; lane 10, transverse colon; lane 11, descending colon; lane 12, liver; lane 14, right lung; lane 15, left lung; lane 16, diaphragm; lane 18, pericardium; lane 19, myocardium; lane 20, aorta. The arrowhead indicates the 16s rDNA amplification product (100 bp). The faster-migrating bands are primer-dimers. Electrophoresis on 3% agarose.

organs. The rationale is that if the body has been heavily colonized by microorganisms from the outer environment (soil, sediments), during its taphonomical history, we expect to find a rather uniform distribution of bacterial DNA in the different organs. Conversely, if the colonization has not taken place, or if it has but not in a massive way, we would expect to find a bacterial DNA distribution reminiscent of that of the living human. With respect to the bowel, for example, we know that under normal conditions the upper part of the small bowel (duodenum, jejunum) has a low microbial content, while its lower portion (ileum) is characterized by higher cell numbers. The highest microbial content is found in the large bowel (colon).

The distribution of low molecular weight bacterial DNA within the internal organs of the Andean mummy is shown in Figure 3. One can observe that the intensity of the amplification signals differs from sample to sample. In particular, we can note that liver, lungs, and diaphragm produce no signal or very weak signals. On the other hand, transverse and descending colon produce very strong signals; comparatively weaker signals are produced by the small intestine and by the ascending colon. This result clearly demonstrates that this mummy has not undergone a massive colonization of microorganisms from the outer environment. The anomalous presence of bacterial DNA in pericardium, myocardium, and aorta is puzzling and could be due to a postmortem reflow of septic blood from the intestine to the heart or to other endogenous causes. The hypothesis that it may be due to penetration of external microorganisms seems less probable as lungs, for example, are shown to contain very little, if any, bacterial DNA. In all cases, the entering of external microorganisms would not have involved the bowel. Quantitation of bacterial DNA using competitive PCR, on the other hand, showed that small intestine and transverse colon of the mummy contained approximately 1,000 and 3,000 copies of low molecular weight (196 bp) 16s rDNA per reaction mixture,

TABLE 1. Characterization of 16s rDNA amplicons from colon (primer pair 29f/98r)

Amplicon	Blast identification	Accession no.	Similarity
2	*Clostridium botulinum* E	L37592	100%
3	*Clostridium botulinum* E	L37592	95%
8	*Clostridium botulinum* E	L37592	98%
24	*Clostridium botulinum* E	L37592	100%
25	*Clostridium botulinum* E	L37592	100%
28	*Clostridium botulinum* E	L37592	100%
29	*Clostridium botulinum* E	L37592	100%
33	*Clostridium botulinum* E	L37592	100%
34	*Clostridium botulinum* E	L37592	100%
44	*Clostridium botulinum* E	L37592	95%
5	*Clostridium botulinum* G	M59087	96%
1	*Clostridium* sp.	X78074	90%
35	*Clostridium* sp.	X95274	96%
6	*Clostridium algidicarnis*	X77676	98%
4	*Eubacterium pectinii*	X67097	74%

respectively, a result that further confirms that the original distribution of the bowel microbial flora is preserved.

DNA cloning and sequencing and DNA sequence analysis

At this point, to determine the taxon composition of the colonic bacterial flora of the mummy, we cloned the 16s rDNA amplification product of the transverse colon sample into a plasmid vector and determined the nucleotide sequence of 15 amplicons. The sequences were then used to scan the EMBL and GeneBank data libraries using Blast and FastA programs. The result is shown in Table 1. Eleven of 15 sequences considered are identified as *Clostridium botulinum*, an organism commonly isolated from soil, marine, and lake sediments. *C. botulinum* (Cato et al., 1986) is found, also, in animal, bird, and fish intestines and in food. The remaining sequences are identified as *Clostridium* sp., *C. algidicarnis* and *Eubacterium pectinii*. We can observe, however, that these identifications must be taken as very circumstantial due to the shortness (approximately 60 bp) of the sequences compared and to the slight similarity to the reference sequences displayed by several of them.

To obtain a more conclusive inference about the relative taxon composition, we amplified the transverse colon DNA preparation, using a different primer pair (338f/531r) designed to bind to a 196 bp long portion of the 16s rDNA. The amplification products were cloned and sequenced as described above (Table 2). We note, incidentally, that attempts to amplify the same DNA type using longer (approximately 350 and 1,500 bp) PCR systems failed.

The taxon list in Table 2 is very different from the previous one. We can observe that, this time, *Clostridium algidicarnis* is the predominant species followed in relative abundance by *C. cochlearium*, *C. aurantibutyricum*, and *C. intestinalis*. *C. algidicarnis* has been primarily described as a psychrotrophic microorganism associated with refrigerated meat spoilage (Lawson et al., 1994). *C. aurantibutyricum* and *C. cochlearium* are found in a variety of substrata including human and animal faeces. *C. intestinalis* has been isolated from animal faeces (Lee et al., 1989) while *C. irregularis* is a component of the normal faecal flora of humans and animals.

Some of the amplicon sequences (Table 2) chosen as representative of the different taxa found have been inserted into a phylogenetic tree of the *Clostridia* (Fig. 4).

DISCUSSION AND CONCLUSIONS

All the papers describing the molecular identification of ancient microorganisms published so far are, with a few exceptions, based on the direct quest for a taxon-specific type of microbial DNA in a set of specimens (see references in Introduction).

The present study is characterized by a substantially novel and more comprehensive approach based on the progressive application of several different tests: amino acid racemization test; mtDNA copy number quantification; bacterial DNA distribution survey. In our approach, taxon identification (based on amplicon sequencing) is the last link in the chain.

For what concerns the last step (taxon identification), we observe that this step poses problems caused by conflicting needs. For example, the ancient DNA doctrine recommends the use of PCR systems targeted to very short DNA regions (around 100–200 bp); this is required to increase the chance of amplifying truly ancient fragments (Handt et al., 1994). On the other hand, taxon identification (starting from mixed microbial populations) in modern environmental specimens is normally based on the

TABLE 2. Characterization of 16s rDNA amplicons from colon (primer pair 338f/531r)

Amplicon	Blast identification	Accession no.	Similarity
1	*Clostridium algidicarnis*	X77676	99%
2	*Clostridium algidicarnis*	X77676	99%
5	*Clostridium algidicarnis*	X77676	96%
17	*Clostridium algidicarnis*	X77676	100%
6	*Clostridium algidicarnis*	X77676	95%
7	*Clostridium algidicarnis*	X77676	95%
11	*Clostridium algidicarnis*	X77676	100%
13	*Clostridium algidicarnis*	X77676	99%
14	*Clostridium algidicarnis*	X77676	100%
26	*Clostridium algidicarnis*	X77676	99%
57	*Clostridium algidicarnis*	X77676	93%
51	*Clostridium algidicarnis*	X77676	97%
31	*Clostridium algidicarnis*	X77676	99%
37	Chimera *(P. aeruginosa + Thermus sp)*	X77676	99%
3	*Clostridium cochlearium*	M59093	99%
60	Chimera *(D. infernum + C. cochlearium)*	M59093	99%
49	*Clostridium cochlearium*	M59093	100%
46	*Clostridium cochlearium*	M59093	97%
33	*Clostridium intestinalis*	X76740	98%
4	*Clostridium intestinalis*	X76740	98%
15	Chimera *(A. enteropelogenes + E. multiforms)*	X76740	98%
9	Chimera *(C. cellulosi + F. nucleatum)*		
19	*Clostridium irregularis*	X73447	95%
12	*Clostridium tetani*	X74770	97%
32	*Clostridium aurantibutyricum*	X68183	99%
18	*Clostridium aurantibutyricum*	X68183	96%
16	*Clostridium aurantibutyricum*	X68183	96%
10	*Clostridium* sp.	X76741	96%
8	*Clostridium* sp.	X76741	96%
47	Chimera *(B. aminovcrans + H. sodomense)*		

comparison of rather long (>300 bp) 16s rDNA sequences.

The screening of two 16s rDNA libraries prepared using PCR systems of different lengths offers an eloquent example of this situation. Taxon identification based on the 29f/98r library (100 bp amplicon length) indicates an almost absolute predominance of *C. botulinum* in the microbial community of the Andean woman's colon. This result, taken as it is, is more than suspicious. On the other hand, we may note that the identification of the bacteria at the genus level (*Clostridium* and *Eubacterium*) is plausible (Hill, 1995).

The 338f/531r library, on the other hand, shows a much higher heterogeneity in taxon composition than the previous one, including *Clostridium* species consistent with the flora of the human and animal colon. We can conclude from this observation that the increase of the target 16s rDNA sequence length allows one to perform an acceptable taxon identification while keeping within the size range of the DNA fragments extracted from the Andean mummy tissues and, in general, within a fragment size range found in most ancient DNA preparations.

One may ask why only 16s rDNA sequences from clostridia are found. There are several possible explanations. The first is that the clostridia are extremely abundant representatives of the anaerobic gut flora. Their proportion is most probably underestimated by the traditional cultivation assays. Second, the clostridia are endospore-forming bacteria and it is known that the spore offers an excellent protection to the DNA (Setlow, 1992). There is the further possibility of an amplification bias (Suzuki and Giovannoni, 1996); this, however, seems unlikely as two different sets of primers both gave consistent results at the genus level. Finally, the prevalence of clostridia may reflect a pathological state (megaly) of this particular colon as shown during the necropsy (Fornaciari et al., 1992).

In conclusion, the present work shows how we can convincingly identify at least some of the major bacterial taxa in the intestinal microflora of a person who lived in the Andes many centuries ago. The protocol

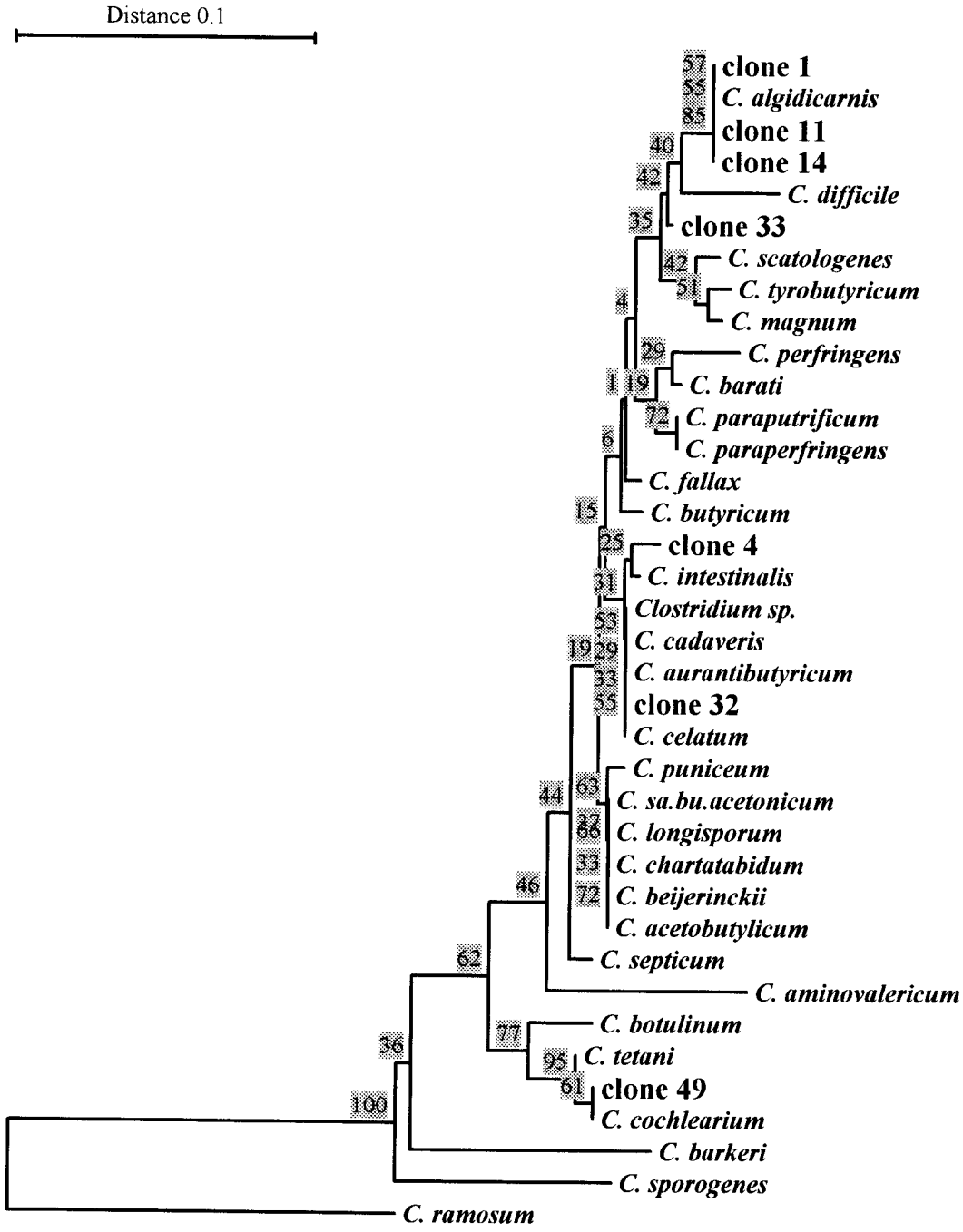

Fig. 4. Bootstrapped (100 replications) 16s rDNA tree of the genus *Clostridium* inclusive of seven amplicon (156 bp) sequences (1, 4, 11, 14, 32, 33, 49) from the Andean mummy colon. The tree was constructed using neighbor-joining (Saitou and Nei, 1987) and the distances were corrected for superimposed mutations according to Tajima and Nei (1984). *C.sa.bu.acetonicum* = *Clostridium saccharoperbutylicumacetonicum*.

employed can be applied to other mummies and can be used to study the resident bacterial flora in ancient humans. As the bacterial flora of the intestinal ecosystem is known to be influenced by diet, interaction among the different groups of microorganisms, drug assumption, toxins, and carcinogenic substances, we potentially have the opportunity to make comparisons among different populations on an ethno-anthropological basis. In addition, also pathogenic species, if present, can be monitored. Salmonellosis, for example, is transmitted from a human to a human without an intermediate host, through fecal contamination of water and food. Serovars adapted to man, e.g., *Salmonella typhi*, *S. paratyphi* A, and *S. sendai*, cause grave diseases with septicemia-typhoidic syndrome (Le Minor, 1984).

Finally, we would like to mention the so-called Tyrolean iceman as a research subject of particular interest for the issues addressed in the present paper. This natural mummy has been dated to 5300–5100 years BP, which corresponds to the Late Neolithic (Spindler, 1995). The Neolithic is generally regarded as demarcating a crucial point in the history of the co-existence of humans and microbes. At that time, the transition to living in large sedentary human aggregates is presumed to have favored the spread of diseases including those characterized by an oro-fecal transmission of pathogens. This is now a testable hypothesis. Studies aimed at identifying the DNA of ancient bacteria preserved within the iceman's intestines are presently being conducted in our laboratories.

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